

MRSA Biosensor Based on CCD Detection

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Abstract

A method for the detection of methicillin resistant *Staphylococcus aureus* (MRSA) using Charge Coupled Device (CCD) detector is described. Monolayers of bacteriophage were formed at an air–water interface and transferred onto silica substrates by Langmuir-Blodgett (LB) method. Firstly, the interactions of a wide host range of *Staphylococcus aureus* lytic bacteriophage and *S. aureus* were characterized on silica substrates by CCD detector. Experiment results indicated that this biosensor system has a detection limit of 10⁵ cfu/ml. To distinguish MRSA and methicillin sensitive (MSSA) strains, a penicillin-binding protein (PBP 2a) specific antibody was used as a secondary probe. A simple agglutination test was carried out using a latex reagent sensitized with monoclonal antibody against PBP 2a. Agglutination indicated the presence of PBP 2a in MRSA strains.

Keywords

Lytic phage; Biosensor; CCD; Phage Monolayer

Introduction

Methicillin resistant strains of MRSA are associated with significant infections and nosocomial outbreaks (Byun et al.; Giamarellou et al.; Knopf). MRSA shows resistance to a wide range of antibiotics thus limiting the treatment options (Broughan et al.; Durgaryan et al.). Recent outbreaks of MRSA in Europe and USA caused panic. They demonstrated lack of approaches to timely recognize dangerous infection and properly handle curing of that disease (Shorr et al.). Currently existing methods of MRSA detection have some limitations and lack sensitivity or specificity. Usually these tests can be used just for the confirmation of methicillin resistance after the specific identification of *S. aureus*, which require at least several hours (Diekema). Therefore, rapid detection of MRSA is of critical importance in prevention and prognosis of infections due to antibiotic resistant *S. aureus*.

In this study, biosensor based on CCD detector was employed for the detection of MRSA using lytic phage probes. Optical biosensors based on CCD detectors provide an expedient method for quantifying bioprobe

and analyte interactions on the biosensor surface. CCD based biosensors have proven to be sensitive and specific for detecting various analytes of interest. Usage of antibodies as molecular probes and biosensors based on CCD were described for detecting avian influenza virus (Qi et al.), filamentous phage M13KO7 (Qi et al.), hepatitis virus (Huang et al.), and *Salmonella typhimurium* (Bae et al.). In the present work, we use lytic phages as probes for recognition of MRSA and CCD to detect bacterial binding.

It was previously demonstrated that bacteriophage 12600 can be used as a recognition probe for *Staphylococcus aureus* strains including those having methicillin-resistance (Balasubramanian et al.; Guntupalli et al.). Recently we demonstrated discrimination of MRSA and MSSA by using specially modified lytic bacteriophage (spheroids) and a penicillin-binding protein (PBP 2a) specific antibody using quartz crystal microbalance (Guntupalli et al.). In present work, we proposed a different approach in specific recognition and detection of MRSA, including identification of bacteria together with the conformation of MRSA in real time. For this purpose, we use an intact unmodified *S. aureus* bacteriophage along with monoclonal antibody against protein (PBP 2a). PBP 2a creates a bacterial cell wall structure and it is responsible for antibiotic resistivity of MRSA. PBP 2a antibody may not recognize MRSA alone as the interaction between PBP 2a protein and antibodies is not specific for *Staphylococcus aureus* and other bacteria have antibiotic binding proteins with large sequence similarity to PBP 2a (Wei et al.). In order to build a biosensor to specifically detect and identify MRSA, we will employ a device with a two-step action. The first step will use an *S. aureus* bacteriophage monolayer as a sensor probe, while the second step will utilize an agglutination test using PBP 2a specific antibodies. Consequently, the step one will identify *S. aureus* bacteria; step two will confirm the presence/absence of antibiotic resistance. It is believed that simultaneous recognition of *S. aureus* bacteria and PBP 2a protein enhances specificity and consistency of MRSA sensing.

Materials and Methods

Staphylococcus aureus lytic phage, bacterial suspensions and chemicals

S. aureus bacteriophage 12600 with a lytic activity against wide spectrum of *S. aureus* hosts, including methicillin-resistant strains, was previously isolated in our laboratory (Balasubramanian et al.; Guntupalli et al.; Guntupalli et al.). Bacterial suspensions for biosensor measurements were prepared in water. The suspensions have been stored at 4°C until the experiment and have been brought to room temperature (25±1°C) before employing in the experimental procedures. The NZY medium was prepared as described (Grieco et al.).

Substrate preparation and phage monolayer deposition

Silica (Si) wafers (WaferNet Inc., San Jose, CA) were cleaned with a base piranha solution (3:1) mixture of ammonium hydroxide (NH₄OH) with hydrogen peroxide (H₂O₂) for 20 minutes at 55°C then thoroughly washed in deionized water and air-dried (Kohler). Si wafers were diced into 20 mm × 10 mm rectangular strips using a diamond tip. Strips were maintained in a desiccator until use and rinsed in hexane prior to LB film deposition. The KSV 2200 LB film balance (KSV Chemicals, Finland), setup, and general procedures for preparing phage monolayers have been previously described (Guntupalli et al.).

Scanning electron microscopy analysis of monolayers

The physical distribution and quality of the monolayers were examined using a JEOL-7000F SEM. Samples were air dried and mounted onto aluminum stubs with copper adhesive tape (Ted Pella Inc. Redding, CA). The images were taken at an accelerating voltage of 5 or 10 kV and a probe current of 54 µA. Working distance was between 10 and 15 mm using a 3 aperture sizes. SEM images of LB monolayers were recorded in the electronic format using JEOL-Imaging software.

Imaging ellipsometer and CCD detectors

A Spectral Imaging Ellipsometer (EP³, Nanofilm Technologies, Göttingen, Germany) with a solid state laser source ($\lambda=532$ nm), a xenon arc lamp with 46 interference filters ($\lambda = 300$ to 1000 nm), and 10× objective with 2 µm spatial resolution and 54° angle of incidence to substrate surface's perpendicular axis, was used for the for surface imaging. All

measurements were carried out at room temperature of 25 ± 2°C with air as the ambient medium. The objective lens and CCD camera combination enabled real-time imaging of illuminated area of the sample (400µm×400µm). In this study, we used CCD camera to quantify phage-bacterial interactions on post assayed samples, and the results were obtained in grayscale format (8 bits, 0–255 grayscale). During the sample analysis, all the conditions were fixed (angle of incidence (54°), Polarizer 22°, compensator 66°, analyzer 78° and wave length ($\lambda=532$ nm)). When all the conditions are constant, CCD detector intensity (I) is a function of analyte layer thickness (d) (Arwin et al.; Huang et al.). Hence the grayscale intensity changes are proportional to the amount of analyte bound to the sensor.

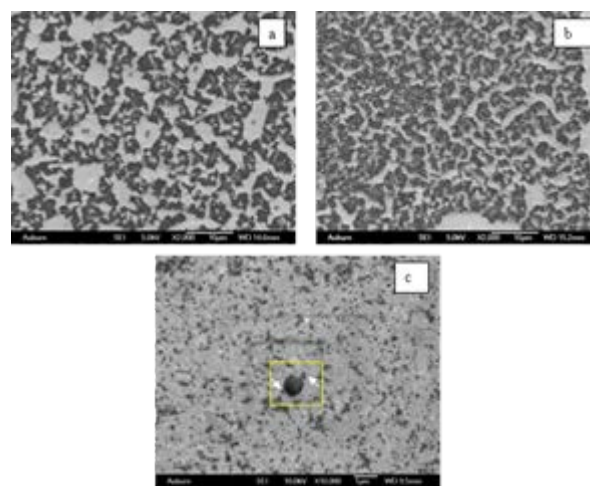


FIG. 1 SEM MICROGRAPH OF LYTIC PHAGE MONOLAYER AND ITS INTERACTIONS WITH MRSA AND *S. AUREUS* BACTERIAL SPECIES, ON A SILICA SUBSTRATE. (A) MRSA BOUND TO THE LYTIC PHAGE IMMOBILIZED ON SILICA SUBSTRATE (×2000) (B) *S. AUREUS* BOUND TO THE LYTIC PHAGE IMMOBILIZED ON SILICA SUBSTRATE (×2000) AND (C) PHAGE MONOLAYER (×10,000). INSET SHOWS THE MRSA CELL BOUND TO PHAGES (1 µm ROUND CELL IS IN THE MIDDLE OF THE INSERT; ARROWS POINT TO PHAGES)

PBP 2a screen test kit

PBP 2a-Screen test was obtained from Denka Seiken Co., Ltd., Tokyo, Japan. The sample preparation was made according to manufacturer's instructions. 10 µl of (10³ to 10⁹ cfu/ml) MRSA (or MSSA) suspension was added to 1.5-ml microtube containing 4 drops (200 µl) of extraction reagent no. 1 (0.1 M NaOH). The suspension was boiled for 3 min, and then 1 drop (50 µl) of extraction reagent no. 2 (0.5 M KH₂PO₄) was added and mixed well. After a centrifugation step (4,500 rpm for 5 min at room temperature), 100 µl of the supernatant was placed on the slide for testing and mixed with 1 drop (25 µl) of anti-PBP 2a monoclonal

antibody sensitized latex. For the negative control, 50 ml of the supernatant was placed on the slide for testing and mixed with 1 drop (25 μ l) of negative-control latex. Mixing for 3 min was performed with the hand using a small plastic stirrer (provided with the kit). The resulting agglutination patterns were visually determined and photographed

Results and Discussions

Characterization of LB monolayers with scanning electron microscopy

We compared immobilized bacteriophage 12600 binding ability against both MRSA and MSSA. Bacteriophage 12600 was immobilized on Silica substrates using LB method as described in materials and methods section. Following this, bacteriophage immobilized substrates were exposed to 10^9 cfu/ml concentration of bacterial suspensions (either MRSA or MSSA). Scanning electron microscopy (SEM) studies were employed to observe the binding ability of bacteriophage 12600 against both MRSA and MSSA post assayed samples. SEM studies revealed that immobilized bacteriophage 12600 is effective in capturing both MRSA and MSSA. Typical SEM images are shown in figure 1a and 1b. Typical SEM images of LB monolayers transferred onto Si strip are shown in Figure 1c. Lytic phage particles are spread homogeneously in the monolayer with estimated density of 8.84 ± 0.6 (N=10) phage particles per square micron or 8.84×10^8 phages/cm² (Guntupalli et al.). Although this corresponds to only $\sim 9\%$ coverage of the total substrate surface, this is twice as large as that obtained using a biotinylated phage immobilization technique (Gervais et al.). Also, it is evident from the figure 1c single bacterium cell is bound to multiple phages (shown in arrows), which may be helpful in improving biosensor performance.

Biosensor based on CCD detector

The biosensor proposed in this study utilizes the principle that formation of a thin film due to bioprobe-analyte interactions would alter the interference pattern of light on the sensor surface. These changes in the interference pattern of reflected light could be quantified by CCD detectors (measures grey image color intensity). Changes in intensity profile were related to the amount of bound target analyte. All the measurements were performed in off-null mode. *S. aureus* bacteriophage was immobilized on silicon substrate by Langmuir-Blodgett method. After

immobilization of phage onto silica substrates, they were exposed to 200 μ l solution of MRSA bacterial cultures for 10 minute to bind bacterial cells. Then these assayed sensors were gently rinsed with deionized water to remove any non-specific binding and air-dried at room temperature.

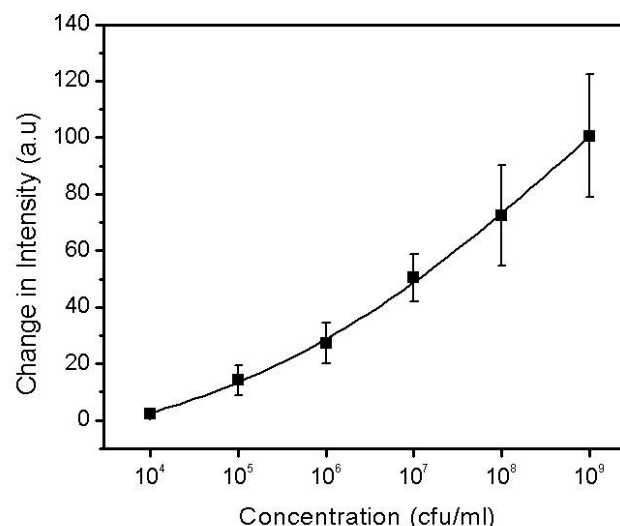


FIG. 2. THE CALIBRATION CURVE FOR QUANTITATIVE DETECTION OF MRSA USING BACTERIOPHAGE 12600. EACH DATA POINT IS AN AVERAGE OF SIX SAMPLES, OBTAINED IN AT ROOM TEMPERATURE UNDER OPTIMIZED CONDITIONS.

At this point, sensors were ready for the CCD analysis and gray scale intensity measurements were performed in the phage immobilized region (devoid of bacteria) and phage-bacterial interactions region. The obtained grey scale intensity response clearly shows that the intensity decreases due to the binding of analyte (MRSA) to the sensor surface. Figure 2 shows a dose dependent MRSA detection calibration curve. The sensitivity for MRSA detection was in the order of 10^5 cfu/ml. Optical microscopy images of post assayed sensors have shown visible difference in the bacterial coverage (data not shown) in terms of cell distribution. After optimizing each step, total assay time could be less than 10 minutes. Each data point in the Figure 2 represents the mean of values obtained from six independent sensors, subjected to study under identical experimental conditions.

Confirmation of MRSA

Visible clumping or agglutination occurred in about three minutes for MRSA strains (Figure 3a) and for MSSA strains no clumping or agglutination occurred (Figure 3b), even after prolonged exposure. Based on our results, MRSA can be detected via latex agglutination kits that act on PBP2a.

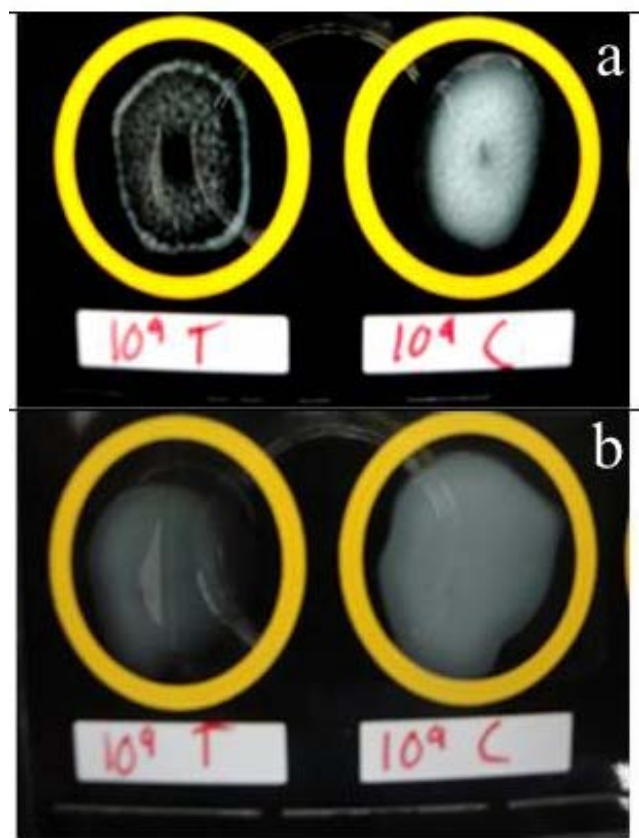


FIG. 3. THE MRSA LATEX AGGLUTINATION KIT WAS EMPLOYED TO DETECT PENICILLIN BINDING PROTEIN 2' (PBP2') IN METHICILLIN RESISTANT STAPHYLOCOCCUS SPECIES. (A) VISIBLE AGGLUTINATION WAS OBSERVED WITH MRSA SPECIES AND (B) NO AGGLUTINATION WAS OBSERVED WITH MSSA SPECIES. IN THIS PICTURE T-REPRESENTS TESTING WITH PBP ANTIBODY COATED LATEX BEADS AND C- REPRESENTS CONTROL TEST, WHERE TESTING WAS CARRIED OUT WITH CONTROL LATEX BEADS (DEVOID OF ANTIBODY)

TABLE 1 AGGLUTINATION PATTERNS WERE DETERMINED BY EYE, WITH THE FOLLOWING RESULTS

Concentration, (cfu/mL)	Test (agglutination level)	Control (agglutination level)
10 ⁹	High	None
10 ⁸	High	None
10 ⁷	High	None
10 ⁶	Medium	None
10 ⁵	Medium	None
10 ⁴	None or not visible	None
10 ³	None or not visible	None

Agglutination was quite visible at concentrations between 10⁵ to 10⁹ cfu/mL. Not much visible agglutination occurred for 10³ and 10⁴ cfu/mL

concentrations of MRSA suspensions. This may be due to not enough presence of PBP 2a protein in the supernatant.

Conclusions

This work has provided a proof of concept for the development of a rapid biosensor technology for the detection MRSA using CCD detectors. *S. aureus* bacteriophage and a penicillin-binding protein (PBP 2a) specific antibody were employed as bioprobes for the specific detection MRSA.

S. aureus bacteriophage immobilized biosensors were fabricated, and their response to different concentrations of MRSA was evaluated. Real time ellipsometric optical images of assayed sensors provided visual verification that the measured grey scale intensity shifts changes due to the attachment of bacteria to the sensor surface.

This biosensor technology has a detection limit of 10⁵ cfu/mL. Methicillin resistance *S. aureus* strains were confirmed with the PBP 2a-Screen test. Visible agglutination occurred in about three minutes for MRSA strains and for MSSA strains no agglutination occurred. This confirms the presence of PBP 2a.

This test proved that simultaneous recognition of *Staphylococcus* bacteria and PBP 2a protein increased specificity and reliability of MRSA detection.

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Dr. Vodyanoy tutored 45 PhD and MS students, authored or co-authored more than 90 refereed scientific publications, 10 book chapters, and produced 54 Patents, (22 US patents, 32 Foreign patents),- 9 of which are currently licensed and commercialized. Some of his awards include the 2000 Pfizer Award for Research Excellence; the 2006 R&D 100 Award; the 2007 R&D 100 Award and 2007 Nano 50 Award.